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Na^+/Na^+ Exchange and Na^+/H^+ Antiport in Rabbit Erythrocytes: Two Distinct Transport Systems

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Summary. Rabbit erythrocytes are well known for possessing highly active Na^-/Na^- and Na^+/H^+ countertransport systems. Since these two transport systems share many similar properties, the possibility exists that they represent different transport modes of a single transport molecule. Therefore, we evaluated this hypothesis by measuring Na^+ transport through these exchangers in acid-loaded cells. In addition, selective inhibitors of these transport systems such as ethylisopropyl-amiloride (EIPA) and N-ethylmaleimide (NEM) were used. Na^+/Na^+ exchange activity, determined as the Na_o^+-dependent ^{22}Na efflux or Na_i^+-induced ^{22}Na entry was completely abolished by NEM. This inhibitor, however, did not affect the H_i^+-induced Na^+ entry sensitive to amiloride (Na^+/H^+ exchange activity). Similarly, EIPA, a strong inhibitor of the Na^+/H^+ exchanger, did not inhibit Na^+/Na^- countertransport, suggesting the independent nature of both transport systems. The possibility that the NEM-sensitive Na^+/Na^+ exchanger could be involved in Na^+/H^+ countertransport was suggested by studies in which the net Na^+ transport sensitive to NEM was determined. As expected, net Na^+ transport through this transport system was zero at different $[Na^+]_i/[Na^+]_o$ ratios when intracellular pH was 7.2. However, at $pH_i = 6.1$, net Na^+ influx occurred when $[Na^+]_i$ was lower than 39 mM. Valinomycin, which at low $[K^+]_o$ clamps the membrane potential close to the K^+ equilibrium potential, did not affect the net NEM-sensitive Na^+ entry but markedly stimulated the EIPA- and NEM-resistant Na^+ uptake. This suggest that the net Na^+ entry through the NEM-sensitive pathway at low pH_i, is mediated by an electroneutral process possibly involving Na^+/H^+ exchange. In contrast, the EIPA-sensitive Na^-/H^+ exchanger is not involved in Na^+/Na^+ countertransport, because Na^+ transport through this mechanism is not affected by an increase in cell Na^+ from 0.4 to 39 mM. Altogether, these findings indicate that both transport systems: the Na^+/Na^+ and Na^+/H^+ exchangers, are mediated by distinct transport proteins.

Key Words Na transport · NEM · amiloride · Na^+/Na^- countertransport · Na^+/H^+ exchange · rabbit erythrocytes

Introduction

Rabbit red cells are well known for the high activity of their Na^+/H^+ exchange (Duhm & Becker, 1979). This ion transport system is sensitive to phloretin and N-ethylmaleimide, is ATP independent, and under normal conditions exchanges external and internal Na^+ in a one-to-one ratio. Because of the latter, its physiological role has been elusive.

The importance of understanding the physiological function of the Na^+/Na^+ countertransport in red cells is based on the finding that its activity is elevated in some groups of hypertensive patients (Canessa et al., 1980; Canessa, 1986). Because similar changes may take place in other tissues as well including vascular smooth muscle, it has been speculated that such alterations could be central in the development of hypertension. Thus, the study of ion transport systems in erythrocytes such as the Na^+/Na^+ countertransport can provide not only an assessment of these transporters but can yield important information on the etiology of this condition.

An important observation regarding the physiological function of this ion transport system was made by Hass, Schooler and Tosteson in 1975. They found that net uphill Li^+ extrusion can be mediated through the Na^+/Na^+ exchanger by using the energy contained in the Na^+ gradient. Nevertheless, since lithium is not a normal constituent of blood plasma, the physiological consequences of this transport mode are unknown. This finding, however, indicated for the first time that the Na^+/Na^+ exchanger could be involved in secondary active transport.

In 1982, Aronson postulated that the Na^+/Na^+ exchanger could operate as an amiloride-sensitive Na^+/H^+ antiport. Indeed, this author has provided evidence indicating the existence of an amiloride-sensitive Na^+/Na^+ exchange mode in the renal Na^+/H^+ antiporter (Aronson, Nee & Suhm, 1982). Grinstein et al. (1984*b*), recently found evidence suggesting the existence of an electroneutral cation exchange system in lymphocytes capable of trans-

porting Na^+ and H^+. However, in contrast to the renal Na^+/H^+ exchanger (Aronson et al., 1982), both Na^+/Na^+ and Na^+/H^+ exchanges occurred through an amiloride-insensitive pathway. Therefore, controversy exists as to whether the Na^+/Na^+ exchanger could operate as a Na^+/H^+ antiporter in other cellular preparations.

Recently, we have provided evidence indicating the existence of substantial Na^+ for H^+ exchange in rabbit erythrocytes (Escobales & Rivera, 1987). This antiporter shares many properties of Na^+/H^+ exchangers in other preparations (Grinstein & Rothstein, 1986) such as: sigmoidal pH_i dependence, inhibition by external protons, hyperbolic activation by external Na^+, a 1 : 1 stoichiometry, electroneutrality, and sensitivity to amiloride or its more potent analogs. However, because in rabbit erythrocytes Li^+/Na^+ or Na^+/Na^+ exchange also appears to be markedly inhibited by H_o^+ and is stimulated by cytosolic acidification (Canessa & Spalvins, 1987), it has been proposed that the amiloride-sensitive Na^+/H^+ exchanger may represent a different transport mode of the Na^+/Na^+ countertransport system (Canessa, 1986). This idea has received support from experiments indicating that in cells with normal Na_i^+ there is a concomitant inhibition of Na^+/Na^+ exchange when the Na^+/H^+ antiport is at maximal velocity (Escobales & Rivera, 1987; Semplicini, Spalvins & Canessa, 1987). Furthermore, preliminary observations in our laboratory indicated that in acid-loaded cells (pH_i = 6.1) the Na^+/Na^+ exchanger is about 50% amiloride sensitive whereas under normal conditions (pH_i = 7.2) this system is amiloride resistant (Escobales & Canessa, 1986).

Jennings, Adams-Lackey and Cook (1985), however, presented evidence against a common pathway for H^+ and Na^+ movements through the Na^+/Na^+ exchanger of the rabbit erythrocytes. They measured Na^+ efflux into Na^+-free or Na^+-containing media under conditions in which an inwardly directed H^+ gradient was imposed. They found that although external H^+ interact with the Na^+/Na^+ exchanger with high affinity, there was negligible transport of protons through this mechanism. Thus, they concluded that H^+ is not a physiological substrate for the Na^+/Li^+ exchanger. However, since H_o^+ are now known to be potent inhibitors of both Na^+/Na^+ (Canessa & Spalvins, 1987) and Na^+/H^+ exchange processes (Escobales & Rivera, 1987), the failure of H_o^+ to promote Na_i^+/H_o^+ exchange through the Na^+/Na^+ countertransport cannot be used as an argument against a common pathway.

Therefore, we decided to evaluate this hypothesis by measuring Na^+/Na^+ and Na^+/H^+ exchange in acid-loaded rabbit erythrocytes incubated at constant external pH. In addition, in order to discriminate between these two transport systems, we used ethylisopropyl-amiloride (EIPA), a highly selective inhibitor of the Na^+/H^+ exchanger (Vigne, Frelin & Lazdunski, 1985) and N-ethylmaleimide (NEM) to inhibit the Na^+/Na^+ countertransport (Duhm & Becker, 1979). The results presented here indicate that these transport systems are mediated by different transport proteins. However, they also indicate that the NEM-sensitive Na^+/Na^+ countertransport system mediate net sodium movements which are electroneutral and EIPA resistant.

Materials and Methods

Materials

NaCl, $MgCl_2$, choline and glucose were purchased from Fisher Scientific (Fairlawn, NY). Ouabain, Tris (TRIS(hydroxymethyl) aminomethane), MOPS (3-[N-morpholino]-propanesulfonic acid), MES (2-[N-morpholino]ethanesulfonic acid), DIDS (4,4'-diisothianocyanatostilbene-2,2'-disulfonic acid), valinomycin and acetazolamide were obtained from Sigma (St. Louis, MO). KCl was obtained from Mallinkrodt (St. Louis, MO). Ethylisopropyl-amiloride (EIPA) was kindly supplied by Dr. E. J. Cragoe from Merk, Sharp and Dohme (Rahway, NJ). Bumetanide was from Laboratoire LEO (Vernouillet, France) and N-ethylmaleimide (NEM) was from Sigma.

Preparation of Red Blood Cells

Blood was obtained by ear vein puncture from New Zealand White male rabbits. The cells were spun down at 3,000 rpm for 15 min at 4°C in a Jouan centrifuge (Model CR 4.11). The plasma and buffy coat were removed by suction, and the cells were then washed three times with a choline washing solution (CHWS) containing (in mM): 150 choline chloride, 1 $MgCl_2$, 10 Tris-MOPS (pH = 7.4 at 4°C). Following this procedure the cells were resuspended to a 75% suspension. Aliquots of this suspension were used for determinations of hemoglobin (optical density at 540 nm), hematocrit and cellular Na^+ and K^+ concentrations by using appropriate dilutions in double-distilled water containing 0.02% Acationox^R detergent (American Scientific Products, Boston, MA).

Internal pH Manipulation and Determination

The procedure described by Escobales and Rivera (1987) was used. Briefly, cells with pH = 6.0 were obtained by preincubating washed cells (8% hematrocrit) for 10 min in media containing in (mM): 145 KCl, 0.15 $MgCl_2$, 10 glucose, 0.1 ouabain, 35 sucrose and 10 Tris-MES (pH = 5.45 at 37°C). Following this incubation

period the cells were spun down at 5,000 rpm for 2 min and resuspended in a media of similar composition to the one mentioned above but with pH set at 6.0 for an additional 10 min. Sucrose was included in the media to counteract the cell volume increase that takes place with acid loading of red cells. Following the final 10-min incubation, DIDS (125 μM) and acetazolamide (1 mM) were added and the cells were incubated for another 30 min. The cells were then spun down, washed twice with its corresponding loading solution without drugs (145 mM choline medium for valinomycin experiments) at 4°C, and kept packed at 80% hematocrit ready for use. For cell with normal pH_i (7.2), the procedure used was similar with the exception that the buffer used throughout was Tris-MOPS (pH = 7.4 at 37°C). A portion of these cells was washed three times with CHWS whose osmolarity was similarly adjusted with sucrose and was resuspended to about 70% hematocrit for determinations of hemoglobin, hematocrit, and cellular Na^+ and K^+ concentrations. This procedure has been shown to maintain normal values of Na^+ and K^+ and cellular volume of pH_i-manipulated red cells (Escobales & Canessa, 1986; Escobales & Rivera, 1987). For pH_i determination, acid-loaded cells were washed twice with 5 volumes of an unbuffered medium and the pellet of cells (90% hematocrit) was lysed with the same volume of double-distilled water. The pH of the lysate was taken to represent the cell pH.

Flux Media

The flux media used in the experiments (unless otherwise indicated) contained (in mM): 145 NaCl, 0.15 $MgCl_2$, 0.1 ouabain, 1 acetazolamide, 0.01 bumetanide, 10 glucose, 35 sucrose, and 10 Tris-MOPS (pH = 8.0 at 37°C) or Tris-MES (pH = 6.0 at 37°C). In the experiments where different concentrations of Na^+ were used, tonicity was maintained with K^+ as replacement. When NEM was used, the drug was added to the media at a final concentration of 0.5 mM. The cells used for NEM experiments were also incubated with the drug (0.5 mM) during the acid loading. EIPA, bumetanide, acetazolamide and NEM were added to the media as required from stocks prepared in dimethyl sulfoxide.

Na^+ Influx Determination

^{22}Na influx was determined as described by Escobales and Rivera (1987). Briefly, rabbit erythrocytes were suspended to 6% hematocrit in the media described earlier (under flux media) at 37°C containing ^{22}Na (1 μCi/ml). At the specified time intervals (1 or 5 min) triplicate samples (1 min) were removed and placed in 1.5-ml Eppendorf centrifuge vials containing 0.2 ml of *n*-butylphthalate. The samples were then spun down immediately for 15 sec at 12,000 rpm in a Fisher microcentrifuge (Model 235C). The aqueous supernatant and the oil layer were removed by suction, the cell pellet was then lysed and aliquots were taken for counting and hemoglobin determinations.

Preparation of Cells for Net Na^+ Transport

To determine net Na^+ movements, $^{22}Na^+$ efflux and influx was measured in cells loaded to contain different concentrations of Na^+. This was achieved by using the nystatin method as described earlier (Escobales & Canessa, 1986). Washed cells were incubated (at 15% hematocrit) with nystatin (40 μg/ml, Sigma) for 20 min at 4°C in a medium containing (in mM): 145 X-Cl (X = Na^+ or K^+), 40 sucrose, 10 Tris-Mops (pH = 7.4 at 4°C) with frequent shaking. After 20 min, the cells were spun down and about 85% of the supernatant discarded. The sample was then split in two portions, one for Na^+ efflux and the other for Na^+ influx. To the cell sample used for Na^+ efflux, $^{22}Na^+$ (1 μCi/ml) was added, and both cell samples were then incubated for an additional 20-min period at 4°C without nystatin. Following this period of time, the cells were transferred to a water bath at 37°C for 10 min after which the cells were washed five times with a media similar in composition to the one used above but containing 1 mM K-phosphate and albumin (1 mg/ml) at 37°C. The cells were then washed three times with a 145-mM KCl medium at 4°C and resuspended to an 80% suspension for acid loading (*see* pH_i manipulation). A portion of these cells was washed three times with CHWS for determinations of hemoglobin, hematocrit, Na^+, and K^+ levels.

Na^+ Efflux Measurements

Acid $^{22}Na^+$-loaded cells were suspended at 10% hematocrit and 37°C in the media described under the flux media section. At specified time intervals (5 min), triplicate samples (1 ml) were taken out and centrifuged for 15 sec at 12,000 rpm in 1.5-ml microfuge vials containing 0.2 ml *n*-butylphtalate. An aliquot of the supernatant was then taken out to determine radioactivity and the cell pellet was discarded.

For Na^+ influx determination in nystatin-treated cells, a procedure similar to that described earlier was used. Net Na^+ transport was estimated from the difference between Na^+ influx and Na^+ efflux. Both Na^+ entry and efflux determinations were made at pH_o = 8.0 and 6.0. Because in both cases determinations at pH_o = 6.0 were very similar to those of the leak pathway (resistant to NEM or EIPA), the values obtained at pH_o = 8.0 were corrected (subtracted) for this flux. Sodium transport through the Na^+/Na^+ exchange pathway in acid-loaded cells is linear for about 15 min. Therefore, a 5-min determination was made. Since valinomycin reduces cell volume, the flux time for the Na^+/Na^+ exchanger determination in these experiments was 3 min. Na^+/H^+ exchange, measured as the H_i^+-induced Na^+ entry, was measured for 1 min to compensate for the high activity rate of this transporter (Escobales & Rivera, 1987). In general, for the cells that required both $[Na^+]_i$ and pH_i to be modified, they were first treated with nystatin and then acid loaded. This procedure did not significantly alter the expected $[Na^+]_i$. Na^+ transport rates were expressed as mmol/liter cells · hr. Data was analyzed using a Paired *t* test analysis.

Cation Measurements

Cellular Na^+ and K^+ concentrations were made by atomic absorption spectroscopy (Perkin Elmer, model 5000) with suitable standards prepared with double-distilled water. The cation content of cells was expressed per liter of original volume.

Table 1. Na^+/Na^+ countertransport in rabbit red cells treated with DIDS and acetazolamide

Conditions	Na^+ efflux (mmol/liter cell · hr)		Na^+ influx (mmol/liter cell · hr)
a) K^+ medium	2.20 ± 0.2		—
	$P < 0.005$		—
b) Na^+ medium	9.68 ± 0.8		—
c) K^- cells	—		4.00 ± 0.1
	—		$P < 0.005$
d) Na^+ cells	—		11.11 ± 0.3
Na^+/Na^+ countertransport	7.49 ± 0.2	NS	7.12 ± 0.2

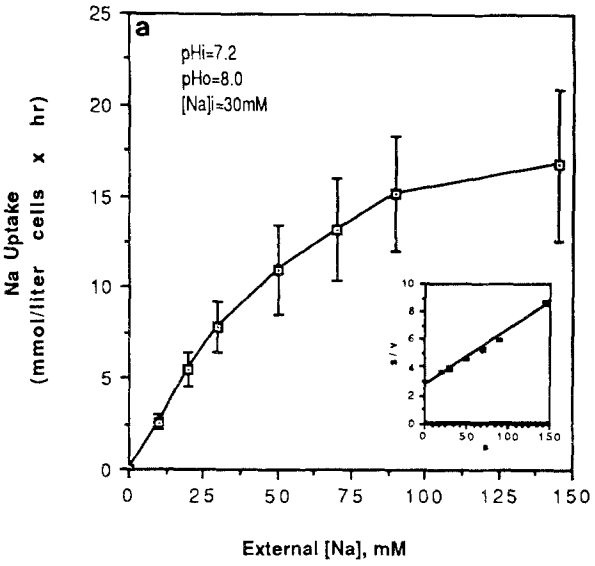
The values shown are the means ± SE of three experiments. Na^+ influx and efflux was measured as described in Materials and Methods. Except for K^- cells, all cells contained 15 mM internal $[Na^+]$. Na^+ concentration in K^+ cells was 0.42 mM. The external $[Na^+]$ and $[K^+]$ was 145 mM. Intracellular pH was clamped at 7.2 by using DIDS (125 μM) and acetazolamide (1 mM), pH_o = 8.0. NS: not different from its control.

Results

Na^+/Na^+ Exchange in Rabbit Red Cells

Na^+ efflux from rabbit erythrocytes into ouabain-containing media is highly dependent on the presence of extracellular Na^+. This transtimulation of Na^+ efflux by external Na^+ is due to the activity of a Na^+/Na^+ countertransport system as found by Duhm and Becker (1979). Therefore, in order to establish the presence of this transporter in these cells, the ouabain-resistant Na^+ efflux ($[Na^+]_i$ = 15 mM) was measured into a K^+ and Na^+ medium with the intracellular pH clamped at 7.2 by using DIDS and acetazolamide (pH_o = 8.0). As shown in Table 1, Na^+ efflux into a K^+ medium was 2.20 ± 0.21 and increased to 9.69 ± 0.83 mmol/liter cell · hr, when Na^+ replaced K^+ in the incubation media, yielding a Na^+-dependent fraction of 7.49 ± 0.6 mmol/liter cell · hr. In order to verify that the Na^+_o-dependent Na^+ efflux represent Na^+/Na^+ exchange activity, the influx into Na^+-free cells ($[Na^+]_i$ = 0.4 mM) was measured under similar experimental conditions. As can be observed in this Table, the fact that both transtimulations (Na^+ efflux by external Na^+ and Na^+ influx by internal Na^+) were of identical magnitude is consistent with the presence of the Na^+/Na^+ exchanger in these cells.

Figure 1*a* and *b* shows the dependence of Na^+/Na^+ exchange activity on external and internal Na^+ concentration. The data conforms to Michaelis-Menten kinetics, with Hanes-Woolf plots yielding



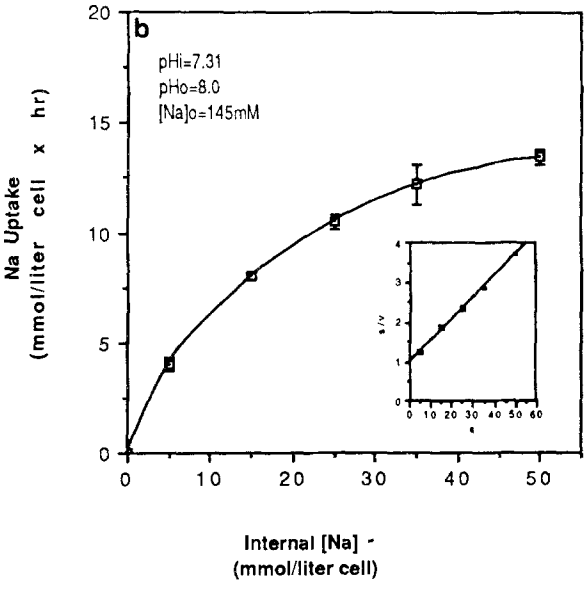


Fig. 1. Na^+/Na^+ exchange activity as a function of external and internal Na^+ concentration. The results shown are the means ± SE of four different experiments in different animals. Na^+ uptake was measured as described in Material and Methods. Cell Na^+ was varied by using the ionophore nystatin. (*a*) Na^+/Na^+ exchange activity was determined as the difference between the Na^+ uptake into a Na^+-containing and a Na^+-free media. (*b*) Na^+/Na^+ exchange activity is estimated as the difference between the Na^+ entry into Na^+-free cells and Na^+-containing cells. The insets show Hanes-Woolf plots of the data (Segel, 1976) yielding approx. affinity constants for internal and external Na^+ of 18.6 and 67.2 mM, respectively. Linear regression equations for the inset in *a* were $Y = 2.6281 + 3.9897e\text{-}2x$, $r^2 = 0.988$, and $Y = 1.0055 + 5.4138e\text{-}2x$, $r^2 = 0.998$ for the inset in *b*

Table 2. NEM inhibits Na^+/Na^+ exchange activity in acid-loaded cells

Internal [Na^+]	Na^+ influx		NEM sensitive
	None	NEM	
	(mmol/liter cell · hr)		
a) 0.42 mM	44.67 ± 1.9	39.83 ± 0.5	4.79 ± 2.6
	$P < 0.01$	NS	$P < 0.025$
b) 39.0 mM	60.84 ± 1.5	40.00 ± 0.5	20.84 ± 1.2
Na^+/Na^+ exchange (b-a)	16.17 ± 1.1	—	16.05 ± 2.4

The values shown are the means ± SE of three experiments. Na^+ influx was measured as described in Materials and Methods. NEM was used at a final concentration of 0.5 mM, and the cells were pretreated with the drug during the acid loading (*see* Materials and Methods). $[Na^+]_i$ was varied by using nystatin. $[Na^+]_o$ = 145 mM. pH_i = 6.1. Influx time was 5 min. NS: not different from its control.

Table 3. Effect of NEM and amiloride on the H_i^+-induced Na^+ entry in Na^+-free erythrocytes

Conditions	Na^+ entry (mmol/liter cell · hr)	Δ
None	75.77 ± 8.3	
		66.77 ± 8.5
Amiloride (1 mM)	9.02 ± 0.9	
		NS
NEM (0.5 mM)	70.41 ± 7.9	
		61.03 ± 7.7
NEM − amiloride	9.37 ± 0.8	

The values shown are the means ± SE of four experiments in different animals. Na influx was determined as described in Materials and Methods. The H_i^+-induced Na^+ entry inhibited by amiloride was taken to represent the activity of the Na^+/H^+ exchange. Na^+ entry was measured in acid-loaded cells (pH = 6.1) at an external pH of 8.0. Influx time was 1 min. $[Na^+]_i$ = 0.42 mM and $[Na^+]_o$ was 145 mM. NS: not statistically different by paired *t* test analysis. Similar effects were obtained when Na^+/H^+ exchange was evaluated by using 20 μM EIPA.

an app. k_m for external and internal Na^+ of 67.2 and 18.6 mM (n = 4), respectively. The results are therefore in close agreement with previous studies on the Na^+/Na^+ countertransport by Duhm and Becker (1979) and Jennings et al. (1985).

Effect of N-Ethylmaleimide (NEM) on Na^+/Na^+ Exchange

Table 2 shows the effect of NEM treatment on Na^+/Na^+ exchange in acid-loaded cells (pH_i = 6.1). As it can be observed, Na^+ influx was stimulated by 16.2 ± 1.1 mmol/liter cell · hr, when internal Na^+ was increased from 0.42 to 39 mM. In NEM-treated cells, however, this transtimulation was completely abolished. Thus, an NEM-sensitive Na^+ entry of 16.05 ± 2.4 mmol/liter cell · hr was found. The fact that the Na_i^+-dependent Na^+ uptake is of similar magnitude to the NEM-sensitive Na^+ entry indicates that this inhibitor is a good estimator of Na^+/Na^+ exchange activity. This Table also shows that the NEM-sensitive NA^+ entry in Na^+-free cells is 4.79 ± 2.6 mmol/liter cell · hr. This effect, however, can be due to the fact that these cells are not absolutely free of Na^+ and therefore the small component of Na^+ entry inhibited by NEM probably represents the residual activity of the Na^+/Na^+ exchanger. If this fraction is taken into consideration, Na^+/Na^+ exchange activity would be 20.84 ± 1.2 mmol/liter cell · hr. This activity which is about 2.8 times higher than that obtained for this transport system at pH_i = 7.2 (*see* Table 1) is indicative of the stimulatory effect of internal H^+ on this exchange as found by Canessa and Spalvins (1987).

Effect of NEM on Na^+/H^+ Antiport

In order to evaluate if NEM is a specific blocker of Na^+/Na^+ exchange its effect on the amiloride-sensitive Na^+/H^+ exchanger of the rabbit red cell was tested. As indicated in Table 3, Na^+/H^+ exchange activity, as measured by the H_i^+- induced Na^+ entry inhibited by 1 mM amiloride ($[Na^+]_i$ = 0.42), was not significantly affected by NEM (from 66.77 ± 8.5 to 61.03 ± 7.7 mmol/liter cell · hr). Similar results were obtained by using the potent amiloride analog ethylisopropyl-amiloride (EIPA, 20 μM) to estimate Na^+/H^+ exchange activity. These findings indicate that NEM can distinguish between these two transport systems.

Is the Na^+/Na^+ Exchange Involved in Na^+/H^+ Countertransport?

If the NEM-sensitive Na^+/Na^+ exchange is also involved in Na^+ for H^+ exchange, the appearance of the latter should be accompanied by an increase in net Na^+ transport sensitive to NEM. To evaluate this possibility, NEM-sensitive Na^+ efflux and influx was measured in cells containing 15 mM Na^+ at pH_i = 7.2 and 6.1, while $[Na^+]_o$ was varied from 5 to 25 mM. The results of this experiment are shown in Fig. 2. As depicted in this figure, net Na^+ movements through the Na^+/Na^+ exchange (pH_i = 7.2) are not different from zero at any combination of internal and external Na^+ concentrated tested. This behavior is to be expected from an operating carrier which exchanges one ion with a 1 : 1 stoichiometry.

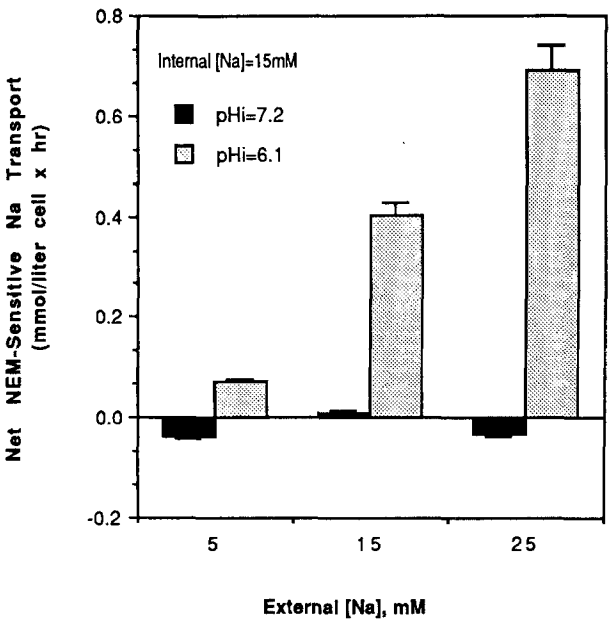


Fig. 2. Net NEM-sensitive Na^+ transport as a function of external Na^+ concentration in normal and acid-loaded cells. The results are the mean ± SE of three different experiments. Net Na^+ transport was measured as described in Materials and Methods

Table 4. Effect of intracellular pH on Na^+ transport through the Na^+/Na^+ exchange system in erythrocytes at variable internal $[Na^+]$

Internal $[Na^+]$ (mM)	pH_i	Na^+/Na^+ exchange		
		Efflux	Influx	Net flux
		(mmol/liter cell · hr)		
15	7.2	7.30 ± 0.04	7.03 ± 0.2	0.27 ± 0.3
		$P < 0.001$	$P < 0.005$	$P < 0.025$
15	6.0	4.87 ± 0.05	10.02 ± 0.01	−5.15 ± 0.8
39	7.2	11.73 ± 1.4	11.60 ± 1.7	0.13 ± 1.8
		$P < 0.025$	$P < 0.025$	NS
39	6.0	24.79 ± 1.0	23.38 ± 1.5	1.41 ± 1.7

The values shown are the means ± SE of three experiments in different animals. Na^+ efflux and influx was estimated as described in Materials and Methods for 5 min. Na^+/Na^+ exchange was estimated as the difference between Na^+ efflux in Na^+-free and Na^+-containing media (145 mM) or between the difference of Na^+ influx in Na^+-free cells (0.4 mM) and cells containing 15 and 39 mM Na^+. Acid loading was performed as described in Materials and Methods. Cell $[Na^+]$ was varied by using nystatin.

Notice, however, that at pH_i = 6.1 a net influx of Na^+ appears as external $[Na^+]$ is increased, reaching a maximal value of 0.77 ± 0.05 mmol/liter cell · hr when $[Na^+]_i/[Na^+]_o$ = 0.6. This net Na^+ entry, which results from a substantial reduction of Na^+ efflux by H_i^+, is consistent with the idea that internal hydrogen may compete with Na_i^+ at its binding site and thus may promote Na_o^+ for H_i^+ exchange.

In the experiment described above net Na^+ transport through the Na^+/Na^+ exchange was estimated by the use of NEM. Therefore, to validate this point, net transport was also determined from the difference between the Na_i^+-dependent Na^+ influx and the Na_o^+-dependent Na^+ efflux at $[Na^+]_i$ = 15 and 39 mM, ($[Na^+]_o$ = 145 mM). As depicted in Table 4, net Na^+ transport was negligible at pH_i = 7.2 at the two $[Na^+]_i$ studied (0.27 ± 0.3 and 0.13 ± 1.8 mmol/liter cell · hr at 15 and 39 mM $[Na^+]_i$, respectively). However, a net Na^+ entry (5.15 ± 0.8 mmol/liter cell · hr) was observed with pH_i = 6.0 at 15 mM $[Na^+]_i$. This net uptake was, however, abolished by an increase in cell Na^+ to 39 mM. This finding at variable $[Na^+]_i$ strongly suggests competition between internal Na^+ and H^+. Thus, as cell sodium is increased the inhibition of Na^+/Na^+ exchange by internal H^+ is abolished.

Effect of Valinomycin at Low $[K^+]_o$ on Net NEM-Sensitive Na^+ Transport

As indicated before the presence of net Na^+ influx through the Na^+/Na^+ exchanger at $[Na^+]_i$ = 15 mM and pH_i = 6.0 is suggestive of the appearance of a Na^+/H^+ exchange transport mode in this transport system. Since it is difficult to measure H^+ fluxes of this magnitude (about 5 mmol/liter cell · hr, at $[Na^+]_o$ = 145 mM), we did not attempt to determine whether there was a detectable Na^+/H^+ exchange in the NEM-sensitive Na^+/Na^+ countertransport system. Nevertheless, because both Na^+/Na^+ and Na^+/H^+ exchanges are electroneutral transport pathways, experiments were done to evaluate the effect that a membrane potential increase (increased inside negativity) would produce on the net Na^+ entry inhibited by NEM. Thus, the K^+ ionophore valinomycin (10 μM) in a low K^+ medium (0.5 mM) was used ($[Na^+]_i/[Na^+]_o$: 15 mM/145 mM pH_i = 6.0). In these experiments the amiloride-sensitive Na^+/H^+ exchange was blocked by using 20 μM EIPA. As shown in Table 5, although valinomycin induced a marked increased in the NEM-resistant Na^+ entry from 7.51 ± 1.45 to 15.28 ± 3.5 mmol/liter cell · hr, the NEM-sensitive fraction of net Na+ entry was not affected (from 6.96 ± 1.0 to 5.94 ± 2.0 mmol/liter cell · hr). This result suggests that in contrast to the NEM-resistant Na^+ entry, the net Na^+ entry sensitive to NEM takes place through an electroneutral process. The latter would be consistent with a Na_o^+/H_i^+ ex-

Table 5. Lack of effect of valinomycin on net Na^+ influx through the NEM-sensitive pathway in the presence of EIPA

Conditions	Net Na^+ entry	
	NEM sensitive	NEM resistant
	(mmol/liter cell · hr)	
None	6.96 ± 1.04	7.51 ± 1.45
	NS	$P < 0.0321$
Valinomycin (10 μM)	5.94 ± 2.04	15.28 ± 3.53

The values shown are the means ± SE of three experiments. NEM-sensitive Na transport was estimated from the difference between Na efflux and influx in the absence and the presence of NEM (0.5 mM). The media contained EIPA (20 μM). $[Na^+]_i$ = 15 mM, $[Na^+]_o$ = 145 mM, pH_i = 6.1, pH_o = 8.0, and $[K^+]_o$ = 0.5 mM.

Table 6. Lack of effect of EIPA on Na^+/Na^+ exchange activity

Internal $[Na^+]$	Na^+ influx		
	None		EIPA
	(mmol/liter cell · hr)		
a) 0.42 mM	56.83 ± 2.7		8.41 ± 0.5
	$P < 0.005$		$P < 0.0025$
b) 39 mM	86.33 ± 4.4		39.18 ± 1.4
Na^+/Na^+ exchange (b-a)	29.50 ± 2.2	NS	30.77 ± 0.8

The values shown are the means ± SE of three experiments. Na^+ influx was measured as described in Materials and Methods. Internal Na^+ concentration was varied by using nystatin. EIPA was used at a final concentration of 20 μM. The Na_i^+-stimulated Na^+ entry was taken as a measure of Na^+/Na^+ exchange activity. $[Na^+]_o$ = 145 mM. Notice that the EIPA-sensitive Na^+ influx (Na^+/H^+ exchange activity) is not affected by the increase in cell Na^+ concentration from 0.4 to 39 mM (from 48.42 ± 1.5 *vs.* 47.15 ± 2.5 mmol/liter cell · hr). Influx time was 5 min. pH_i = 6.1, pH_o = 8.0 NS: not statistically different from its control.

change mode in the Na^+/Na^+ countertransport system.

Is There Na^+/Na^+ Exchange Through the Na^+/H^+ Antiporter?

The previous suggestion concerning the existence of an electroneutral, Na^+ for H^+ exchange in the NEM-sensitive Na^+/Na^+ countertransport prompted us to evaluate whether the amiloride-sensitive Na^+/H^+ antiporter mediates Na^+/Na^+ countertransport. As a first step to evaluate this possibility, experiments were carried out to verify that EIPA did not affect the NEM-sensitive Na^+/Na^+ exchange. Table 6 illustrates the results of experiments in which Na^+ influx into acid-loaded cells (pH_i = 6.1) was determined in virtually Na^+-free ($[Na^+]_i$ = 0.42 mM) and Na^+-containing cells (39 mM) in the presence and absence of 20 μM EIPA. As indicated in this Table, the Na^+/Na^+ exchange is not affected by EIPA (from 29.5 ± 2.2 to 30.77 ± 0.8 mmol/liter cell · hr). In addition, notice that the EIPA-sensitive Na^+ influx (amiloride-sensitive Na^+/H^+ exchange) did not vary when $[Na^+]_i$ was increased from 0.42 to 39 mM (from 48.42 ± 1.5 to 47.15 ± 2.5 mmol/liter cell · hr). Furthermore, Table 6 shows that the induction of Na^+/Na^+ exchange by the rise in $[Na^+]_i$ at 145 mM Na_o^+, is not associated with a concomitant decrease in Na^+/H^+ countertransport. Similar results are obtained when the net EIPA-sensitive Na^+ transport is determined (N. Escobales and J. Figueroa, *unpublished data*). These findings indicate that the EIPA-sensitive Na^+/H^+ exchange do not mediate Na^+/Na^+ countertransport.

Discussion

The results presented here strongly support the idea that the Na^+/Na^+ countertransport and the Na^+/H^+ exchange of rabbit red cells are mediated by two distinct transport systems. Thus, Na^+/Na^+ countertransport was fully inhibited by NEM, a sulfhydryl agent, while the Na^+/H^+ exchange was insensitive to this agent. In contrast, Na^+/H^+ exchange was completely abolished by the potent amiloride analog EIPA. The latter had no effect on the Na^+/Na^+ countertransport. The high selectivity of these two drugs in inhibiting these two transport systems is consistent with their independent nature.

NEM and EIPA are known to act at two different sites. Thus, whereas amiloride (EIPA), appears to bind to the outward-facing transport site of the Na^+/H^+ exchanger (Aronson, 1983; Grinstein et al., 1984*a*; L'Allemain, Paris & Pouyssegur, 1984), the NEM binding site appears to be located on the cytoplasmic site of the Na^+/Na^+ countertransport (Grinstein, Cohen & Rothstein, 1985). Indeed, in order to completely abolish the erythrocyte Na^+/Na^+ countertransport with NEM (0.5 mM), the cells had to be treated and then incubated in the presence of this drug. This is in contrast to EIPA for its presence in the incubation media suffices to produce a rapid and strong inhibition of Na^+/H^+ exchange.

It is important to point out that the selective inhibition of Na^+/H^+ exchange induced by EIPA is not shared with its parent compound amiloride (0.2 mM), or with 0.03% glutaraldehyde, an amino-reac-

tive agent which locks the exchanger in the "off" or "on" mode (Parker, 1984). Thus, these latter drugs affected both the Na^+/Na^+ countertransport and the Na^+/H^+ exchanger (N. Escobales and J. Figueroa, *unpublished results*). This indicates that these agents are not specific inhibitors of Na^+/H^+ exchange in red cells. The findings of this study concerning the lack of effect of NEM on Na^+/H^+ exchange is in contrast with the study of Grinstein et al., (1985) in thymic lymphocytes showing significant inhibition of this antiporter by this agent. Since the inhibitory effect of NEM on Na/H exchange activity is reduced at acid pH_i (Grinstein et al., 1985), this discrepancy probably results from the lower intracellular pH used by us.

A conclusion derived from this work is that the EIPA-sensitive Na^+/H^+ exchanger in red cells is not involved in Na^+ for Na^+ countertransport. Thus as indicated in Table 4, when $[Na^+]_i$ was increased from 0.42 to 39 mM the Na^+/Na^+ exchange activity increased from 10.02 ± 0.1 to 23.38 ± 1.5 mmol/liter cells · hr, but the EIPA-sensitive Na^+ entry was not affected (Table 6). These findings indicate the absence of Na^+ for Na^+ countertransport in the Na^+/H^+ exchange. Similar results were found by Semplicini et al. (1987) in human red cells. In contrast, our data is consistent with the idea that the Na^+/Na^+ countertransport system of the rabbit red cells may also perform Na^+ for H^+ exchange. Thus, Na^+/Na^+ countertransport mediates net Na transport at low internal $[Na^+]$ and pH (Fig. 2 and Table 4). The latter is not affected by a membrane potential increase by using valinomycin at low $[K^+]_o$, suggesting its electroneutral nature. Although electroneutral, net Na^+ entry may occur in exchange for cations such as K^+ or as a symport with anions such as Cl^-, the most likely candidate to be exchanged for external Na^+ is internal H^+. This stems from the fact that an increase in cell $[H^+]$, at low cell $[Na^+]$ is central in the induction of net Na^+ transport. However, since H^+ efflux studies were not conducted to verify a Na^+/H^+ exchange mode in the Na^+/Na^+ countertransport system, additional experiments are needed to settle this important point.

Canessa and Spalvins (1987) were the first to report that internal acidification also stimulates Na^+/Na^+ countertransport. The results presented here confirm this finding. Recently, preliminary experiments done in our laboratory indicate that $[H^+]_i$ has a biphasic effect on Na^+/Na^+ countertransport. Thus, this transport system was markedly activated (from 1.9 to 72.1 mmol/liter cell · hr, $n = 2$) when pH_i was decreased from 7.2 to 6.4 ($[H^+]_{50} \approx 125$ nM). However, when pH_i was further reduced to 6.2, countertransport activity was reduced to 37.4 mmol/liter cell · hr. In contrast, in the same cellular preparation Na^+/H^+ exchane did not vary when pH_i was decreased from 7.2 to 7.11 (from 4.8 to 4.5 mmol/liter cell · hr, respectively), but increased linearly when pH_i was reduced from 6.8 to 6.2 (from 13.4 to 75.6 mmol/liter cell · hr, respectively). Since the $[H^+]_{50}$ values for activation of Na^+/Na^+ countertransport appear to be significantly lower than those for Na^+/H^+ antiport activation, 275 nM (Escobales & Rivera, 1987), it seems of utmost importance to evaluate if the capacity of the Na^+/Na^+ countertransport to carry out net transport of sodium is enhanced, at normal $[Na^+]_i$, in the pH_i range from 7.2 to 6.8.

The marked difference in behavior between the EIPA-sensitive Na^+/H^+ antiport and the NEM-sensitive Na^+/Na^+ countertransport presented in this study are clear indications of the independent nature of both of these transporters. These results are in agreement with those of Grinstein et al. (1984*b*) indicating the existence of an amiloride-resistant Na^+/Na^+ antiport which can also perform Na^+ for H^+ exchange in thymic lymphocytes. In these cells the Na^+/Na^+ exchange differ from the amiloride-sensitive antiport in its pH_i and temperature sensitivity. The Na^+/Na^+ exchange, however, is not similar to that present in rabbit red cells as the lymphocyte exchanger mediates amiloride-insensitive Na^+/H^+ countertransport at normal pH_i (7.0). Our findings are in contrast to those of Aronson et al. (1982), indicating the existence of Na^+/Na^+ exchange in the amiloride-sensitive Na^+/H^+ antiporter in acid-loaded membrane vesicles from kidney tubules. Therefore, it appears that marked variability exists concerning Na^+/Na^+ and Na^+/H^+ exchange systems in different cellular preparations. The latter weakens the possibility of using these transport systems in red cells as a means of assessing their activities in other cell types (i.e., smooth muscle cells).

In summary, our evidence indicates that rabbit red cells possess two distinct ouabain-resistant countertransport systems involving Na^+. One is the EIPA-sensitive Na^+/H^+ exchanger which is activated by a decrease in cellular pH and the other is the Na^+/Na^+ countertransport inhibited by NEM. The NEM-sensitive transport system is also activated by internal H^+, and perform electroneutral net Na^+ transport at low internal Na^+ and pH. Since the latter property of the Na^+/Na^+ countertransport is critical to understand its physiological role, additional experiments are necessary to elucidate whether Na^+ for H^+ exchange is mediated by this transport system.

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